Intracellular Metabolism of Human Apolipoprotein(a) in Stably Transfected Hep G2 Cells†

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ABSTRACT: Lipoprotein(a) [Lp(a)] consists of LDL and the glycoprotein apolipoprotein(a) [apo(a)], which are covalently linked via a single disulfide bridge. The formation of Lp(a) occurs extracellularly, but an intracellular assembly in human liver cells has also been claimed. The human apo(a) gene locus is highly polymorphic due to a variable number of tandemly arranged kringle IV repeats. The size of apo(a) isoforms correlates inversely with Lp(a) plasma concentrations, which is believed to reflect different synthesis rates. To examine this association at the cellular level, we analyzed the subcellular localization and fate of apo(a) in stably transfected HepG2 cells. Our results demonstrate that apo(a) is synthesized as a precursor with a lower molecular mass which is processed into the mature, secreted form. The retention times of the precursor in the ER positively correlated with the sizes of apo(a) isoforms. The mature form was observed intracellularly at low levels and only in the Golgi apparatus. No apo(a) was found to be associated with the plasma membrane. Under temperature-blocking conditions, we did not detect any apo(a)/apoB-100 complexes within cells. This finding was confirmed in HepG2 cells transiently expressing KDEL-tagged apo(a). The precursor and the mature forms of apo(a) were found in the ER and Golgi fractions, respectively, also in human liver tissue. From our data, we conclude that in HepG2 cells the apo(a) precursor, dependent on the apo(a) isoform, is retained in the ER for a prolonged period of time, possibly due to an extensive maturation process of this large protein. The assembly of Lp(a) takes place exclusively extracellularly following the separate secretion of apo(a) and apoB.

Lp(a) represents a macromolecular complex in human plasma which is assembled from LDL[1] and the Lp(a)-specific glycoprotein apolipoprotein(a) [apo(a)] (for comprehensive reviews, see refs *1* and *2*). High plasma concentrations of Lp(a) have been reported as a strong risk factor for premature atherosclerosis and stroke in numerous case/control and prospective studies (*3*, *4*). The apo(a) locus determines almost exclusively the largely varying plasma concentrations of Lp(a) (*5*, *6*); a large proportion (50−70%) of this variation can be attributed to the apo(a) size polymorphism (*7*, *8*).

Human plasma Lp(a) originates exclusively from synthesis in the liver (*9*). Apo(a) is homologous to plasminogen and consists of an inactive protease domain, a kringle V domain and multiple repeats (*2*−*35*) of kringle IV domains (*10*). The size of apo(a) is determined by the number of kringle 4 repeats (*11*, *12*). Lp(a) concentrations correlate negatively with the apo(a) size. In vivo turnover studies in humans have shown that this correlation is based on the apo(a) size-dependent production rate and is not due to different catabolism (*13*). From these studies, however, it is not clear whether different production rates reflect different rates of translation off the ribosome or different export rates. The precise mechanisms of the apo(a)-size-dependent secretion from the human liver are unknown. Transcriptional but also translational or even posttranslational mechanisms are possible and have been reported (*14*, *15*).

In vitro studies in baboon hepatocytes and HepG2 cells have focused on posttranslational mechanisms that influence the Lp(a) production rate (*16*, *17*). These studies have shown that apo(a) is synthesized as a precursor with smaller size. After an unusually long and apo(a) isoform-dependent retention in the endoplasmic reticulum, apo(a) is processed (by O- and N-linked glycosylation) to its mature form (*18*). It was, however, recently demonstrated in the baboon hepatocyte model that the pattern and kinetics of apo(a) folding intermediates did not differ between allelic apo(a) variants (*19*). Apo(a) required 30−60 min to reach its fully oxidized form. The authors suggested that trimming of N-linked sugars, which occurs after folding, is required for secretion, and this processing step might be dependent on apo(a) isoform size.

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; FCS, fetal calf serum; HBS, HEPES-buffered saline; LDL, low-density lipoproteins; ME, mercaptoethanol; NADPH, nicotine amide-adenine-dinucleotide-phosphate; PMSF, phenylmethanesulfonyl fluoride; PNS, postnuclear supernatant; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TGN, *trans*-Golgi network; TRITC, tetramethylrhodamine isothiocyanate.

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Another issue of the cell biology of apo(a) synthesis is the question of where and how assembly of the mature Lp(a) occurs. Does binding between the apoB and apo(a) moieties take place intracellularly, e.g., in the ER as is the case for many oligomeric proteins (*20*, *21*), or only after secretion of the lipoprotein-free apo(a) within the plasma compartment? Evidence for (*22*, *23*) and against (*24*−*29*) an intracellular assembly of Lp(a) has been provided by various authors using several cell systems, animal models, and analytical techniques.

Since previous studies did not investigate the intracellular pathway of newly synthesized human apo(a) and therefore cannot exclude an intracellular Lp(a) assembly in human hepatocytes, we decided to study in more detail the precise intracellular metabolism of recombinant apo(a) in transfected HepG2 cells.

MATERIALS AND METHODS

Transfected HepG2 Cells. Human hepatocarcinoma HepG2 cells were stably transfected by electroporation with plasmids encoding apo(a) isoforms with 10, 14, and 22 kringle IV repeats (24). Cells were cultured in minimal essential medium (DMEM, high glucose) with Earle's salts supplemented with 10% (v/v) FCS, 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, and 400 μg/mL G 418.

Transient transfections with plasmids pCMV-A18 and pCMV-A-18-KDEL into HepG2 cells were performed with the lipofectamine reagent (GIBCO-BRL) as previously descibed (*17*, *24*). Both plasmids encode 18 kringle apo(a) isoforms, which differ by the presence of a carboxyterminal ER retention signal (SGKDEL) (*30*).

Homogenization and Subcellular Fractionation of HepG2 Cells. Cells were grown to 70% confluency in 14 cm Petri dishes and washed twice with ice-cold PBS. They were then gently removed with a rubber policeman, centrifuged for 5 min at 750 rpm and 4 °C, and resuspended in 3 mL of homogenization buffer. After centrifugation for 10 min at 2500 rpm and 4 °C, the pellet was resuspended in homogenization buffer including 0.5 mM EDTA. Cells were homogenized in a glass homogenizer with several strokes. After each stroke, 2 μL of homogenate was withdrawn and checked by light microscopy. Homogenization was stopped after 85−90% of cell nuclei were cell-free. A postnuclear supernatant (PNS) was obtained by centrifugation of the homogenate for 10 min at 3000 rpm and 4 °C.

Subcellular fractionation of the homogenate was performed by density step-gradient ultracentrifugation in the flotation mode (*31*). Briefly, 500 μL of PNS was adjusted to a density of 40.6% sucrose by adding 500 μL of 62% sucrose. To this 1 mL of PNS, 1.5 mL of 35% sucrose and 1 mL of 25% sucrose were overlayered and filled with homogenization buffer including EDTA. Ultracentrifugation was performed in a Beckman rotor SW 56 for 90 min at 35 000 rpm and 4 °C; 500 μL fractions were collected.

The separation of subcellular fractions was monitored by measuring marker enzymes for the endoplasmic reticulum (ER) and the Golgi complex. We chose the NADPH-cytochrome *c* reductase activity as the marker for the ER (*32*); β-galactosyltransferase served as the marker for the Golgi complex (*33*).

Immunofluorescence Labeling of Transfected HepG2 Cells. Stably transfected HepG2 cells were analyzed by immunofluorescence labeling using a monoclonal antibody (5A5) against apo(a) that does not react with catalase or other intracellular proteins (data not shown). Plasma membranes were stained by immunolabeling with a polyclonal rabbit antibody against the α-subunit of Na^+K^+-ATPase (kindly provided by Kaethi Geering, Lausanne, Switzerland). Nontransfected HepG2 cells served as controls. Cells were grown on cover slips for 2−4 days until they reached subconfluency and fixed for 5 min each at −20 °C with methanol and acetone. Before and after 5 min incubation at room temperature of the fixed cells with the primary antibody (in PBS), cells were washed three times for 3 min with PBS. TRITC-labeled secondary antibodies were preadsorbed on fixed cells for 30 min and finally centrifuged in an Eppendorf centrifuge for 5 min at high speed.

Homogenization and Subcellular Fractionation of Human Liver Tissue. Human liver tissue was obtained from resections of liver tumor operations with the permission of the local ethics committee. Tumor-free liver tissue (1 g) was placed immediately into ice-cold homogenization buffer (0.25 M sucrose, pH 7.4, containing 0.1 mM PMSF, 0.1 mM leupeptin, 100 KIU/mL aprotinin, and 3 mM imidazol). The tissue sample was cut into small pieces of approximately 1 mm^3, washed five to six times with homogenization buffer, and homogenized with two strokes in a Teflon-piston homogenizer at a concentration of 1 g of liver tissue/3 mL of buffer. PNS was obtained by centrifugation of the homogenate for 10 min at 2000 rpm at 4 °C and then subjected to density gradient ultracentrifugation on a 15−40% metrazimide gradient (in homogenization buffer) at 30 000 rpm and 4 °C in a VTi 65.1 vertical rotor (Beckman Instruments, Palo Alto, CA) (*34*). Marker enzymes for endoplasmic reticulum (ER) and Golgi complex were measured in subcellular fractions (*32*, *33*). EDTA plasma from the same patient was obtained by low-speed centrifugation.

Metabolic Labeling and Pulse-Chase Experiments. In 3.5 cm dishes, 1.6×10^6 cells/mL of medium were grown for 20 h followed by 2 × 15 min culture in methionine-free medium. The cells were then metabolically labeled by incubating with 250 μCi of [^{35}S]methionine/mL of methionine-free medium for 16 h. After pulse-labeling, medium was removed, replaced with fresh medium, and chased for 0.5−4 h. At given times, medium was removed and cells were lysed by detergent treatment. Briefly, cells were washed twice with Hepes buffered saline (HBS, 50 mM Hepes, and 200 mM NaCl, pH 7.5), incubated for 5 min with HBS + 0.5 M iodoacetamide, and lysed by adding 1 mL of lysis buffer (HBS, containing 2% CHAPS, 1 mM PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, and 10 mM iodacetamide). The cell lysate was cleared of debris by centrifuging for 4 min at 10 000 rpm.

Temperature Block Experiments. Stably transfected HepG2 cells expressing apo(a) isoforms with 14 repeats were metabolically labeled for 16 h with 500 μCi of [^{35}S]methionine/mL in methionine-poor (10%) MEM culture medium and chased in MEM (supplemented with 10% FCS) at 15 °C (block at the ER level), 20 °C (block at the TGN) and 37 °C (no block, regular secretion) for 6 h (*35*, *36*). Cell culture

medium was collected, and cells were lysed as described above.

Immunoprecipitation. Samples of medium and lysates were incubated by rotating for 2 h with 100 μL of Immunoprecipitin (formalin-fixed Staphylococcus aureus cells from GIBCO-BRL) and then centrifuging for 2 min at 10 000 rpm. The respective supernatants were then incubated by rotating for 16 h at 4 °C with 5 μL of a polyclonal anti-apo(a) antiserum raised against purified apolipoprotein(a) (*37*). Immunoprecipitin (100 μL) was added and further incubated at 4 °C for 2 h. After centrifuging at 10 000 rpm for 1 min, the pellets were washed five times with 400 μL of washing bufffer (20 mM MOPS, 20 mM NaH_2PO_4, pH 7.2, 5 mM EDTA, 1% Triton X-100, and 0.5% sodium-desoxycholate) and dissolved in sample buffer for electrophoretic analysis.

Electrophoretic Procedures. Samples were analyzed by reducing or nonreducing SDS−PAGE using either 6.6% polyacrylamide gels according to Neville et al. as previously described (*34*) or commercially available precast 4% Tris-Glycine polyacrylamide gels according to the manufacturer's protocol (NOVEX, San Diego, CA). Immunoblotting was performed according to the standard protocol of Towbin et al. (*38*) using monoclonal anti-apo(a) antibody 1A2 (*34*) and chemiluminescence detection. Metabolically labeled proteins were visualized by fluorography after fixation of the gels in 7% acetic acid and 20% methanol. Fluorography was performed with commercially available solutions (Entensify, NEN, Boston, MA). Protein concentrations were determined according to Bradford (*39*) with commercially available reagents (Biorad, Richmond, CA).

RESULTS

Subcellular Fractionation and Localization of Apo(a) in Transfected HepG2 Cells. HepG2 cells secrete apolipoprotein B containing lipoproteins but no apo(a)/Lp(a) (*40*). We therefore used for our studies on the intracellular metabolism of apolipoprotein(a) HepG2 cells that were stably transfected with plasmids containing constructs of differently sized apo(a) (K10, K14, K22, expressing apo(a) with 10, 14, and 22 kringle IV repeats, respectively). Figure 1 shows immunoblots after reducing SDS−PAGE of intracellular and secreted forms of apo(a) from HepG2 cells expressing apo(a) with two different sizes (K10 and K22). Postnuclear supernatants (PNS) predominantly contained an apo(a) form with a lower molecular mass [pr-apo(a)] and, to a much lesser extent, a mature form identical in size to the secreted form in the medium. To assign the two intracellular forms to distinct intracellular compartments, PNS from two different cell lines (K10 and K22) was separated into subcellular fractions by sucrose density ultracentrifugation, and the distribution of apo(a) in these fractions was determined by immunoblotting. Fractions containing the endoplasmic reticulum (ER) and the Golgi complex were identified by marker enzymes. This experiment revealed large amounts of pr-apo(a) in the ER, whereas a comparably smaller amount of the mature form was localized exclusively in the Golgi fractions. This form was identical in size to the secreted apo(a) in the cell culture medium. Golgi fractions also contained substantial amounts of pr-apo(a), possibly due to incomplete separation between ER and Golgi compartments.

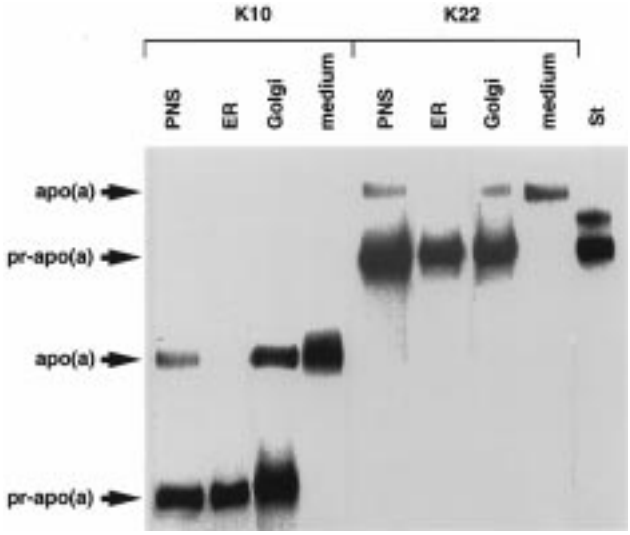

FIGURE 1: Intracellular and secreted forms of apo(a) in HepG2 cells stably transfected with apo(a) constructs of two different sizes. HepG2 cells were stably transfected with plasmids expressing apo(a) isoforms with 10 or 22 kringle IV repeats (K10 and K22). Cells were cultured, homogenized and a postnuclear supernatant (PNS) obtained by low-speed centrifugation as described in the Materials and Methods. PNS was separated into subcellular fractions by sucrose density gradient ultracentrifugation. PNS and fractions with the highest activities in ER and Golgi marker enzymes were subjected, together with the respective medium, to 4% SDS−PAGE (NOVEX) under reducing conditions, followed by immunoblotting with the anti-apo(a) monoclonal antibody 1A2. St represents a plasma standard with apo(a) isoforms 18/20.

No apo(a) immunoreactivity was observed in the PNS pellet containing most of the plasma membrane (data not shown). The subcellular distribution of precursor and mature apo(a) was different for both apo(a) isoforms, consistent with the results obtained in pulse-chase experiments (see Figure 4).

Immunofluorescence Labeling of Apo(a) in Transfected HepG2 Cells. To further investigate the intracellular localization of apo(a), transfected cells were fluorescence-immunolabeled with anti-apo(a). As can be seen from Figure 2A, immunolabeling was preferentially observed in the perinuclear region of the transfected cells. Peripheral cellular regions were only weakly stained, whereas the plasma membrane was completely unstained. These results thus agreed well with our previous analysis of the predominant subcellular localization of apo(a) in the ER. Immunodetection with anti-Na-K-ATPase was performed to label the plasma membrane (Figure 2C), whereas nontransfected cells served as negative control (Figure 2B).

Apo(a) in Human Liver Tissue. To also confirm the intracellular localization of apo(a) in native human liver cells, human liver tissue of a patient with high plasma Lp(a) concentrations and a single expressed apo(a) isoform 23 was homogenized and separated into subcellular fractions, followed by SDS−PAGE and immunoblotting (Figure 3). In the plasma, a single apo(a) isoform was detected, whereas in the liver homogenate two apo(a) species were observed. The larger form had the same apparent molecular mass as the apo(a) found in plasma. Subcellular fractionation localized the lower molecular mass apo(a) form to the ER, whereas the high molecular mass (plasma) form was found only in Golgi fractions.

Pulse-Chase Experiments with Stably Transfected HepG2 Cells. Pulse-chase experiments were performed to demon-

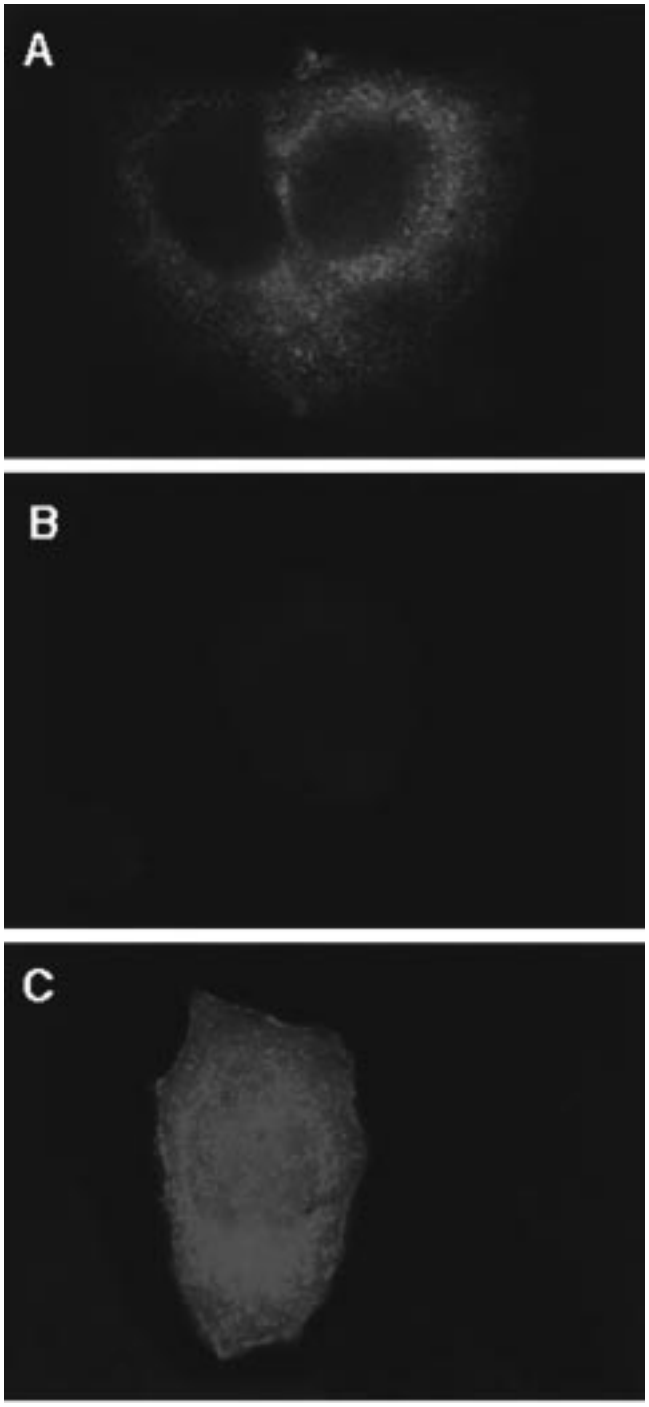

FIGURE 2: Immunofluorescence labeling. Stably transfected HepG2 (K22) cells (2A) and nontransfected control HepG2 cells (2B) were analyzed using monoclonal anti-apo(a) antibody 5A5. In a third experiment, transfected HepG2 cells were labeled with anti−Na^+K^+-ATPase to stain the plasma membrane (2C).

strate the precursor−product relationship between the two intracellular apo(a) forms and to investigate whether the kinetics of intracellular apo(a) processing differ between stably transfected HepG2 cells synthesizing apo(a) with various isoforms.

Figure 4, panels A−C, shows the maturation of the smaller intracellular apo(a) form [pr-apo(a)] into the secreted form during a chase period of up to 4 h for three different apo(a) constructs (K10, K14, and K22) in a representative experiment. Within this chase period, a decrease of pr-apo(a) in cell lysates and a concomitant increase of mature apo(a) in the medium was observed for all apo(a) constructs. In Figure 4, panels D and E, this conversion is quantified by densitometry and expressed as the decline of the precursor and the increase of the mature form in the medium versus time. The $T_{1/2}$ values for the apo(a) isoform precursors K10, K14, and K22 were about 1.0, 1.5, and 2 h, respectively. Thus, a significant positive correlation between size of apo(a) isoforms and the retention time was revealed ($p < 0.05$, $R = 0.997$, Pearson). Likewise, the increase of the mature apo(a) form in the culture media was apo(a) isoform dependent as well. Smaller apo(a) isoforms were more rapidly secreted into the cell culture medium than were larger isoforms. The apo(a) isoform size thus correlated inversely with the conversion rate from pr-apo(a) into apo(a).

Temperature Block Experiments. When samples of PNS and culture media (as shown in Figure 1) were analyzed under nonreducing conditions, a high molecular mass protein band, corresponding to an apo(a)−apoB complex, was observed only in apo(a) immunoblots from culture supernatants, but not in the intracellular homogenates (Figure 5). This suggested that no protein complex forms prior to secretion. The experiment does not, however, completely rule out an intracellular assembly since secretion of an apo(a)−apoB complex might be a much faster process compared to other maturation steps within the cell. In this case, this complex might simply not be observed within the cell.

To address this issue we incubated the transfected cells under temperature conditions known to inhibit the secretory pathway at the ER-to-Golgi step (15 °C) or at the level of exit from the trans-Golgi network (TGN, 20 °C) (*35*, *36*). Figure 6 shows a representative experiment using an apo(a) construct with 14 kringle IV repeats. Metabolically labeled cells were chased for 6 h at 15, 20, or 37 °C, lysed and subjected to nonreducing SDS−PAGE together with samples from their respective culture supernatants. No high-molecular mass apo(a)−apoB complex was detected intracellularly under any conditions of temperature block. Substantial amounts of such a complex were found only at 37 °C in the culture medium. The blocking conditions led to an almost complete inhibition of apo(a) secretion into the medium. After chasing for 6 h, about half of the pr-apo(a) was converted into the mature apo(a) when the secretory pathway was blocked at the TGN level. This suggests that no apo(a)−apoB complex is formed in apo(a)-transfected HepG2 cells.

HepG2 Cells Transfected with KDEL-Tagged Apo(a). To extend the above findings with a different strategy for blocking apo(a) secretion, we transiently transfected HepG2 cells with a plasmid encoding an apo(a) isoform with 18 kringles and the C-terminal ER-retention signal KDEL. For comparison, HepG2 cells were transfected with the same construct without the tag (wild-type, WT). Seventy-two hours following transfection, cells were fractionated by density gradient ultracentrifugation. Figure 7, panels A and B, shows the analysis by reducing SDS−PAGE/immunoblotting of PNS and subcellular fractions (*1*−*8*) of culture medium (M) from HepG2 cells transfected with KDEL-tagged and wild-type apo(a). No apo(a) was detected in the culture medium of cells transfected with KDEL-tagged apo(a) (panel A). Accumulation of the KDEL-tagged apo(a) precursor in the ER (fractions 1−3) was much more pronounced as compared to the wild-type cells (panel B).

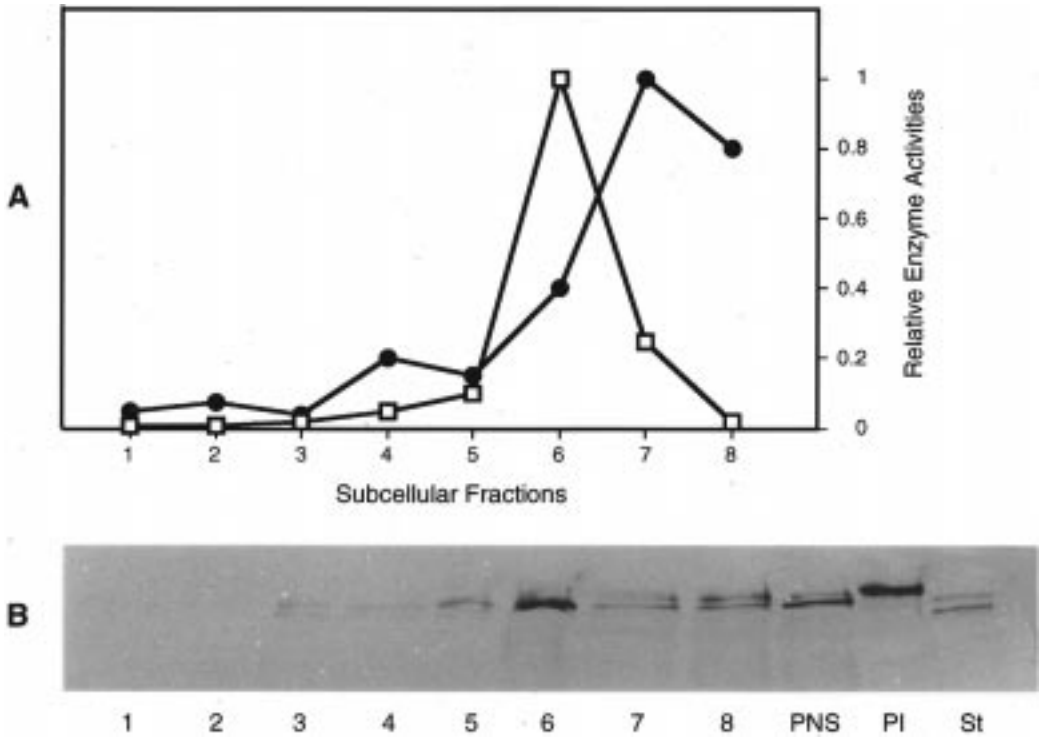

FIGURE 3: Immunoblot analysis of subcellular fractions from human liver tissue. (A) PNS of human liver tissue was obtained after low speed centrifugation of the tissue homogenate and subjected to density gradient ultracentrifugation in a metrazimide gradient. Fractions containing endoplasmic reticulum (ER) and Golgi complex were identified by measuring their respective marker enzymes NADPH-cytochrome reductase (open squares) and β-galactosyl transferase (closed circles). Panel B shows an immunoblot with anti-apo(a) antibody 1A2 of subcellular fractions 1−8, PNS, plasma of the same individual (Pl) and a plasma standard with apo(a) isoforms 18/20 (St) after 6.6% SDS−PAGE under reducing conditions.

When PNS and culture supernatants from cells transfected with the KDEL-tagged apo(a) were analyzed under non-reducing conditions by SDS−PAGE/immunoblotting, we again did not detect a high molecular mass apo(a)-apoB complex in the cell homogenate (Figure 7C).

DISCUSSION

These studies investigated the secretory pathway of apo(a) in human liver cells and revealed three major results: (i) a single apo(a) isoform is present intracellularly in two forms in stably transfected HepG2 cells and in human liver tissue that can be localized to ER and Golgi fractions, respectively. The lower molecular weight form is highly abundant in the ER and represents the precursor of the mature form, which, at much lower abundance, is found exclusively in the Golgi. The differences in molecular weight are probably only apparent and reflect various mobilities due to a different glycosylation pattern [as previously shown by treatment with tunicamycin (*40*), monensin (*41*) and glycosidase (*18*)] in various cell systems. Taken together, our results confirm and extend earlier findings in transiently transfected HepG2 cells (*17*) and primary baboon hepatocytes (*18*). In contrast to the latter cell model, no plasma membrane-associated apo(a) could be detected in stably transfected HepG2 cells. (ii) Pulse-chase experiments showed a precursor−product relationship of the two forms and demonstrated a varying, apo(a) size-dependent intracellular retention of apo(a) secretion, the rate of which was negatively correlated with the apo(a) isoform size. (iii) No intracellular formation of an apo(a)−apoB complex was observed in transfected HepG2 cells, either under conditions where intracellular trafficking was blocked by reduced temperatures or artificial ER retention or when induced. We can therefore definitely exclude an assembly of Lp(a) within the cell prior to secretion in this cell system.

The results of our study suggest an intriguing mechanism for the regulation of secretion of a human secretory protein: the exclusively genetically controlled Lp(a) plasma concentrations are obviously controlled (at least to a major extent) by an apo(a) isoform-dependent retention in the endoplasmic reticulum. This isoform-dependent maturation and secretion is in agreement with studies by White et al. in the baboon primary hepatocyte model (*18*) as well as by Brunner et al. in transiently transfected HepG2 cells (*17*) and extends this concept also to human HepG2 cells that were stably transfected with apo(a) DNA constructs differing only in the copy number of the kringle IV domain. In our hands, transiently transfected HepG2 cells are not suitable for metabolic labeling and pulse-chase experiments, possibly due to slow synthesis rates of apo(a). Stably transfected cells, in contrast, allow extensively long labeling times and therefore represent a very useful model system mimicking the genetic size heterogeneity of the apo(a) molecule in human populations.

In contrast to human apo(a), the molecular genetics of the apo(a) size heterogeneity in baboons has never been elucidated. The underlying mechanisms of the isoform-dependent apo(a) expression in baboon hepatocytes might, therefore, not be comparable to that of human hepatocytes. The transient association between apo(a) and the plasma membrane prior to its secretion, which was found in baboon hepatocytes (*29*) but not in this study, appears to be one such

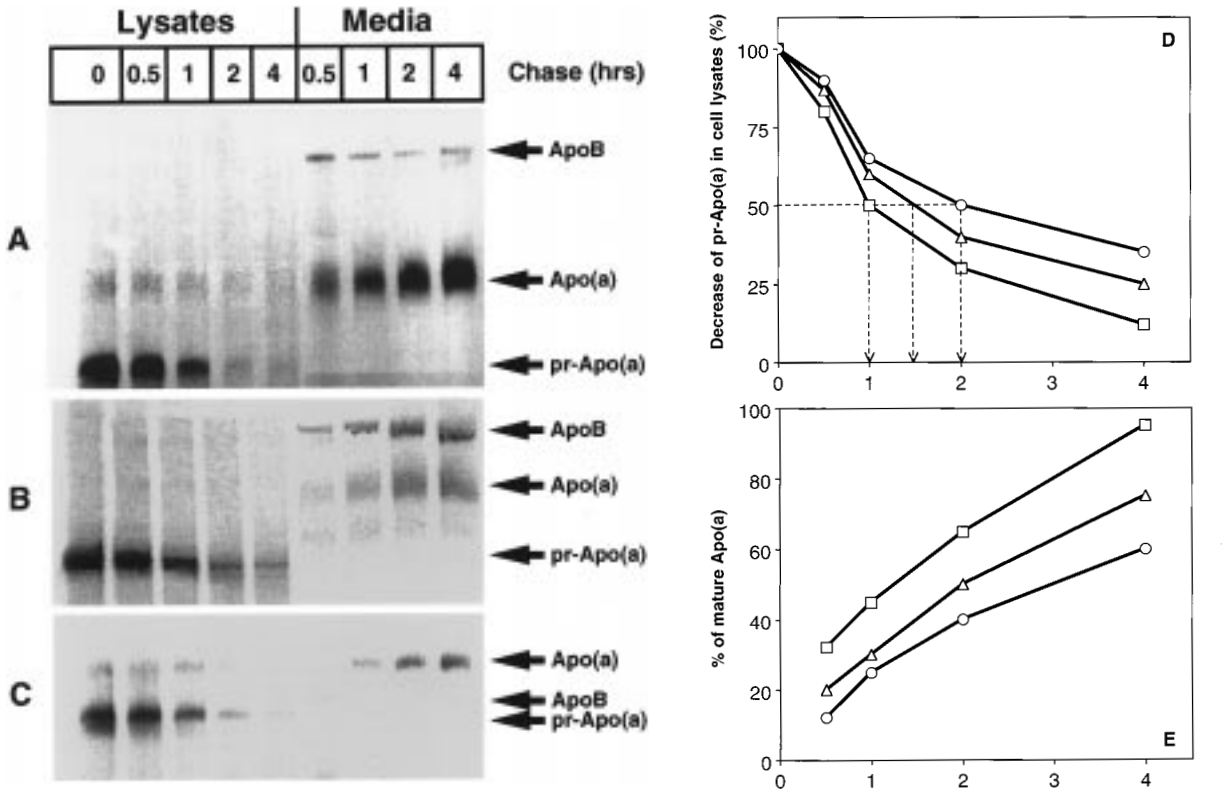

FIGURE 4: Metabolic labeling of and pulse-chase experiments with HepG2 cells transfected with apo(a) constructs of different sizes. Stably transfected HepG2 cells expressing apo(a) with 10 kringle IV repeats (K10, panel A), 14 kringle IV repeats (K14, panel 4B) and 22 kringle IV repeats (K22, panel 5C) were incubated with 250 μCi [^{35}S]methionine for 16 h. After a chase of 0.5, 1, 2, and 4 h, cells were lysed. Apo(a) was immunoprecipitated from cell lysates and media and separated using 4% SDS−PAGE under reducing conditions. Equal amounts of protein were applied to each lane. In vivo labeled proteins were visualized by fluorography. The respective positions of apo(a) precursor [pr-apo(a)], mature apo(a), and apoB are indicated by arrows. Panels D and E show the densitometric evaluation of the pulse-chase experiments presented in panels 4A−C. The decrease of intracellular pr-apo(a), which is calculated relative to the band intensity at the start of chase (100%), is shown in panel D. Squares indicate values for K10 cells, triangles for K14 cells and circles for K22 cells. Arrows indicate the times ($t_{1/2}$) at which the labeled intracellular pr-apo(a) reached 50% intensity relative to the value at the beginning of chase. $T_{1/2}$ was determined to be 1 h for K10 cells, 1.5 h for K14 cells, and 2 h for K22 cells. Panel E shows the increase in secreted apo(a) in the medium. The values are expressed as relative amounts of the mature apo(a) form in the medium compared to the total of intracellular pr-apo(a) and secreted apo(a). The small amount of intracellular mature apo(a) was neglected.

example of a difference between the two species. In HepG2 cells, the extracellular assembly obviously takes place without the involvement of the plasma membrane, in accordance with previous work demonstrating Lp(a) assembly in cell-free systems (*41*). We must, however, take into consideration that our findings might represent a HepG2-specific phenomenon and that in human hepatocytes apo(a) could transiently associate with the plasma membrane, similar to baboon hepatocytes.

For at least 10 years, it has been generally accepted that the concentration of a secretory protein is usually much higher in the Golgi apparatus than in the endoplasmic reticulum (*42*, *43*). The kinetic basis for these observations has been called the "bulk flow hypothesis" (*44*), which states that secretory proteins, after being translocated into the ER lumen, are further transferred by vesicular transport from the ER to the Golgi and secretory vesicles at a default rate. There is, however, increasing evidence from several investigations that proteins of different size, folding, and degree of glycosylation are secreted from HepG2 cells at different rates (*45*−*48*). Various degrees of quality control and processing during the maturation of these proteins could account for these findings. Our data clearly depict a different intracellular distribution of a secretory protein: apo(a) is predominantly localized in its immature form in the ER, whereas comparatively little (mature) apo(a) can be found in the Golgi (Figure 1). In light of the above-mentioned concept, the intracellular maturation process of apo(a) can therefore be considered rather unusual.

Our experiments finally demonstrate through several approaches that in HepG2 cells the Lp(a) complex is assembled only after separate secretion of its constituents. The site of this assembly has been a controversial subject for many years. Recombinant apo(a) from transfected CHO cells is able to assemble to Lp(a) in the culture medium with exogenously added human LDL involving Cys^{4057} of apo(a) (*41*). Extracellular Lp(a) assembly was also confirmed by studies in mice transgenic for human apo(a), which were infused with human LDL (*28*). When investigating cell lysates and culture supernatants under nonreducing conditions, most authors were unable to observe apo(a)−apoB complexes in cell lysates and therefore concluded that Lp(a) is assembled only after secretion (*24*, *41*). This assumption, however, did not take into account the fact that secretion of a putative intracellularly formed Lp(a) might be a rapid process, compared to the intracellular processing of apo(a), and subsequently may have been overlooked in these experiments. A similar argument could be raised against the data of Wilkinson et al. (*27*). Using various sets of ELISA measurements, this group quantified the amount of

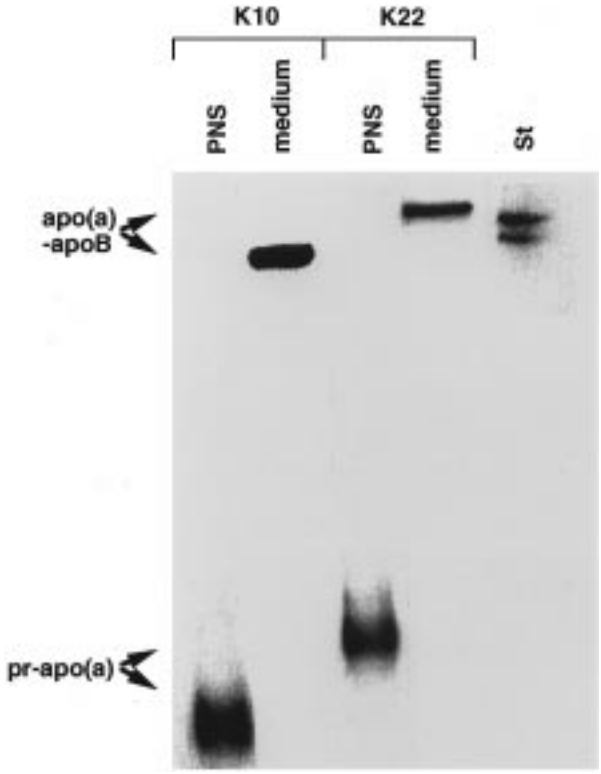

FIGURE 5: Immunoblot analysis of apo(a) from stably transfected HepG2 cells following SDS−PAGE under nonreducing conditions. PNS obtained from cell homogenates of HepG2 cells (K10 and K22), together with the respective cell culture medium, was separated using 4% SDS−PAGE under nonreducing conditions and detected by immunoblotting with anti-apo(a) monoclonal antibody 1A2. St represents a plasma standard with apo(a) isoforms 18/20.

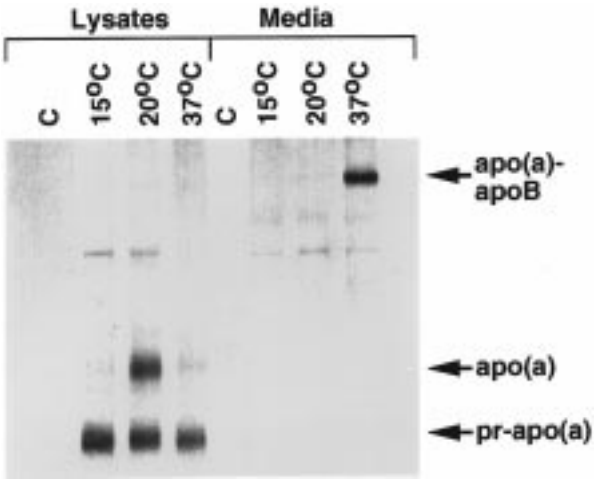

FIGURE 6: Metabolically labeled transfected HepG2 cells, chased at various temperatures. HepG2 cells (K14) were incubated with 500 μCi [^{35}S]methionine in methionine-poor medium for 16 h. After a chase of 6 h at three different temperatures (15, 20, and 37 °C), cells were lysed and, together with the respective cell culture media, immunoprecipitated with polyclonal anti-apo(a) antibody. Precipitates were subjected to 4% SDS−PAGE under nonreducing conditions and analyzed by fluorography. Lysates and media from nontransfected HepG2 cells served as control (C). Arrows indicate the positions of pr-apo(a), apo(a), and the high molecular weight apo(a)−apoB complex.

apo(a) in free or apoB-bound form in homogenized biopsies from human liver tissue. Since no apo(a)−apoB form was detected with this approach, it was concluded that apo(a) is not coupled to apoB. If assembled Lp(a) is secreted rapidly,

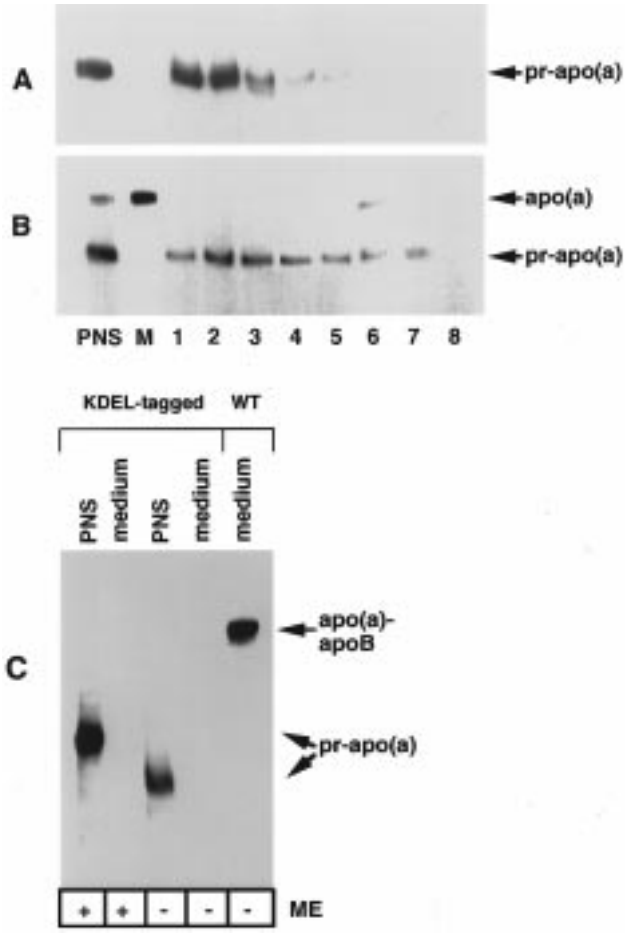

FIGURE 7: Immunoblot analysis of apo(a) from HepG2 cells transiently transfected with KDEL-tagged apo(a). HepG2 cells were transiently transfected with a plasmid containing apo(a) with 18 kringle IV repeats (K18) tagged with the ER retention signal sequence KDEL (panel A). As a control, HepG2 cells transiently transfected with a wild-type apo(a) (K18, WT) were investigated (panel B). Cells were homogenized 72 h after transfection and the PNS obtained by low-speed centrifugation. PNS was separated into subcellular fractions by sucrose density gradient centrifugation. PNS, cell culture medium (M), and subcellular fractions (lanes 1−8) were separated using 4% SDS−PAGE under reducing conditions and immunoblotted with anti-apo(a) antibody 1A2. Precursor [pr-apo(a)] and mature forms of apo(a) are indicated by arrows. In panel C, PNS and medium from HepG2 cells expressing KDEL-tagged apo(a) were separated with 4% SDS−PAGE under reducing (+ME) and nonreducing (−ME) conditions. Medium from HepG2 cells expressing the wild-type apo(a) (K18 and WT) was applied as a positive control. Arrows indicate the precursor apo(a) [pr-apo(a)] as well as the apo(a)−apoB complex form.

then the intracellular Lp(a) concentration might be too low for detection in this system.

The elegant work of White et al. in the primary baboon hepatocyte model elucidated that Lp(a) is assembled extracellularly exclusively after secretion of apo(a) (*29*). When adding antibodies against apo(a) and apoB to the cell culture medium, the authors could not detect any assembled Lp(a), which excludes a preexisting, intracellularly derived covalently linked apo(a)−apoB complex. Similar experiments were also performed in transfected HepG2 cells and led to the same results (*26*). Noncovalent intracellular associations between apo(a) and apoB, however, could not be investigated with such an experimental approach.

The literature, however, also contains reports suggesting an intracellular assembly of Lp(a) in humans. Edelstein et

al. found varying amounts of apo(a)−apoB complexes in lysates from primary human hepatocytes (*22*). Surprisingly, and in contrast to other studies, no intracellular precursor of apo(a) was detected in their cell lysates. Very recently, an intracellular apo(a)−apoB complex formation was also observed in HepG2 cells transfected with an apo(a) minigene (*23*). The experimental evidence for the existence of an intracellular complex was, first, Lp(a) formation despite the presence of anti-apo(a) antibodies, second, coimmunoprecipitation of apo(a) and apoB from cell lysates, and third, the recovery of Lp(a) from microsomal fractions. Interestingly, this Lp(a) consisted of precursor and mature apo(a) as well. Nevertheless, the physiological relevance of these findings remains questionable since the transfected apo(a) consisted of only the signal sequence, six nonidentical kringle IV domains, the kringle V and the protease domain. An Lp(a) particle with such a small apo(a) has never been observed in humans and may form a conformation different from a native particle. Thus, it can hardly be considered a suitable model system for Lp(a) assembly.

We therefore set conditions in our experiments with transfected HepG2 cells, under which any apo(a)−apoB complexes would be detected, if present. In none of our experiments [temperature block, KDEL-tagged apo(a)] was an intracellular apo(a)−apoB formation observed. Although the secretion of apo(a)/Lp(a) could not be blocked completely at the restricted temperatures [which is a common phenomenon in such experiments (*49*)], it was inhibited to a high degree so that any potential intracellular Lp(a) formation would have been detected. Theoretically, intracellular protein assembly might take place in secretory vesicles and would then have been overlooked in our experiments where the secretory pathway was blocked at 20 °C. This possibility is very unlikely, however, since studies conducted under these conditions with viral proteins have shown these proteins to accumulate in the TGN and in vesicular structures destined for transport to the plasma membrane (*35*, *36*).

Comparing Lp(a) formation with other, similar oligomerizations, it is rather surprising that HepG2 cells do not use their sophisticated intracellular folding and quality control machinery to assemble a complicated macromolecule like Lp(a). The endoplasmic reticulum would be expected to provide, with a set of chaperones including calnexin, its suitable redox state and, most importantly, with appropriate enzymes for disulfide formation (e.g., protein disulfide isomerase, PDI), all the necessary tools. The question therefore arises, what conditions keep the two constituents apo(a) and LDL apart within the cell and make them assemble only after secretion? One explanation might be the conformation of the larger partner in this association, namely LDL. It is known from several studies that apoB-containing lipoproteins (like other lipoproteins) are modified along their secretory route not only in the ER but also in the Golgi complex (reviewed in ref *50*). It might be that, without that final maturation, the LDL particle is not in the correct conformation to assemble with apo(a). The above-mentioned intracellular assembly in HepG2 cells transfected with an apo(a) minigene (*23*) argues, however, against an influence of the conformation and composition of the LDL-like particle in HepG2 cells. Thus, the question of what keeps apo(a) and LDL apart to assemble intracellularly, remains obscure.

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